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13. ABSTRACT (Maximum 200 Words)

Breast cancer is one of the most prevalent human cancers, and a leading cause of death among women. BRCA2 is associated with 30-40% of all hereditary cases, predisposing women to early onset breast cancer. The activity of this gene/protein has mainly focused on its involvement in the processes of DNA repair and recombination. We performed a yeast two hybrid to identify new interacting proteins that may indicate its involvement in other cellular processes. Using a human mammary cDNA library we found three independent clones of the gene tristetraprolin (TTP) to interact with BRCA2. This interaction was mapped to an 80 amino acid stretch of the BRCA2 protein using a yeast interaction mating analysis approach. We next transfected HEK-293 cells with epitope tagged constructs (TTP-FL) to demonstrate an *in vivo* association. Initial results proved successful in demonstrating this interaction. However, for unknown reasons, this interaction ceased to occur. Despite a step-wise procedure to recreate the original *in vivo* results, we were still unable to generate a positive result.

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Introduction

Breast cancer is one of the most prevalent human cancers and a leading cause of death among women. Germline mutations in the breast cancer susceptibility gene 2 (BRCA2) are associated with 30-40% of hereditary breast cancer cases and recently, biallelic BRCA2 mutations were shown to be responsible for complementation group D of Fanconi Anemia[1,2]. Much of the research has focused on BRCA2 and its involvement in DNA repair due to cellular sensitivity to DNA damaging agents in BRCA2-null mice[3,4]. The association of BRCA2 with DNA has also been explored involving studies with RAD51 and DSS1, proteins shown to interact not only with BRCA2 and DNA but, as well, in combination[5,6]. To address this possibility of BRCA2 involvement in other cellular processes we assayed a large portion of the BRCA2 protein in two yeast two hybrids. Human and murine mammary cDNA libraries yielded, among others, three independent clones coding for human tristetraprolin gene (TTP) and seven independent clones coding for the murine Limd1 gene. TTP is involved in the degradation of TNF-α mRNA[7] which is underscored by TTP-null mice having high levels of TNF-α, and the manifestation of autoimmune-like complications as a result of these elevated levels. These symptoms were alleviated by the injection of antibodies towards the TNF-α. It is also noteworthy that the BRCA2 promoter contains an Nf-kB responsive element, one of the major genes/proteins activated upon cellular exposure to TNF-alpha.

The purpose of this proposal was to validate the proposed interaction of BRCA2 and TTP *in vivo* and, if direct, *in vitro* and to further physically map the location of this association. As well, to explore the implications of a BRCA2/TTP interaction on the levels of TNF-alpha, a potent cytokine.

Body

Year 1(2002-2003)

Task 1. The confirmation of the BRCA2/TTP protein interaction using in vitro and in vivo model systems and to further define the minimal region responsible for interaction (1-12 months).

First year tasks were accomplished and reported in the candidates Annual Army Report from 2003. Briefly, a smaller area of interaction between BRCA2 and TTP was mapped using subclones of the original BRCA2 bait. Interaction mating analysis in yeast indicated the interaction occurred in an 80 amino acid stretch near the N-terminus of the original bait (Figure 1a). Initial transient transfection studies in HEK-293 cells demonstrated a coimmunoprecipitation between BRCA2 and TTP (Figure 1b).

Year 2(2003-2004)

Task 2. Analysis of TTP mRNA and protein levels using murine cells (12-24 months)

Subsequent to the submission of the first annual report the interaction between BRCA2 and TTP from the 293 cells ceased. This resulted in a reworking of the original Statement of Work and Tasks in order to consistently generate this interaction.

Re-worked Task 2.

Re-confirm the BRCA2/TTP interaction *in vivo*

After the submission of the annual report in 2003 the interaction between BRCA2 and TTP ceased to occur with a typical negative blot seen in Figure 2a. Since we had not demonstrated this association in other cells, it was necessary to re-establish this result in HEK-293 cells.
We took a step-by-step approach to identify the problem.

- Lysis buffer: New batches of the original lysis buffer (NETN) were mixed from fresh supplies. This also included purchasing new protease inhibitors however, this did not reconstitute the interaction. Further attempts were carried out using different types of lysis buffers based on protocols from papers that had previously published a TTP interaction[8,9]. These included buffers with higher and lower stringencies compared to the original NETN. Again, none of these proved successful in restoring the interaction.
- HEK-293: At the time of the loss of interaction we thawed a new aliquot of frozen 293 cells due to incubator contamination with yeast. After establishing these cells, a coimmunoprecipitation was carried out. This did not result in the restoration of the interaction. We decided to order a fresh HEK-293 cells from ATCC and re-try the coimmunoprecipitation with these new cells. Again, numerous attempts did not result in a restoration of the interaction.
- Leptomycin B (LMB): Leptomycin B is a compound that blocks the CRM1-dependent protein export from the nucleus. Tristetraprolin harbours the CRM1-dependent sequence, and in cells that are exposed to LMB, accumulates within the nucleus[10]. Since all published evidence indicates that BRCA2 is strictly contained within the nucleus, we hypothesized that exposing 293 cells to LMB may facilitate the interaction. Taken further, it was postulated that the association may be transiently occurring within the nucleus, and that by blocking TTP export from the nucleus to the cytoplasm (where the predominate amount of TTP is located), we could visualize the interaction. As with previous attempts, this failed to restore the interaction (Figure 2b).

In Vitro Analysis of a direct interaction between BRCA2 and TTP

In addition to the efforts to reestablish the *in vivo* association, we carried out S^{35} experiments to ascertain whether there was direct interaction between BRCA2 and TTP. BRCA2 is too large a protein to generate *in vitro* therefore, MYC-tagged peptides of BRCA2, based on the sequences from the yeast interaction mating analysis (see Figure 1a) were produced. Peptide F-3-1#6 is the postulated TTP interaction site on BRCA2 and B-M2 represents the extreme C-terminus of BRCA2 and was included as a control (Figure 3). Although we did demonstrate an in interaction between F-3-1#6 and TTP, it was diminished by the fact that TTP also interacted with BRCA2 peptide B-M2, a construct that was shown not to interact in the yeast interaction mating analysis. We could not draw any concrete conclusions from this analysis.

Key Research Accomplishments

Year 1

- A proposed interaction between BRCA2 and TTP emanating from a yeast two hybrid assay using a human mammary cDNA library.
- Fine-mapping region within BRCA2 responsible for the association with TTP to a stretch of 80 amino acids (from an original 1300 amino acid bait).
- *In vivo* coimmunoprecipitation of TTP-FLAG in transfected HEK 293 cells.

Year 2

- A methodical approach to reconstitute the interaction observed in the first year. Unfortunately, all attempts were able to achieve this result.

Reportable Outcomes

2002 AACR Annual General Meeting. San Francisco, CA.

Huggins, C.J. and Andrulis I.L. Characterization of a proposed novel BRCA2 interaction

2003 AACR Annual General Meeting. Toronto, ON.

Huggins, C.J. and Andrulis I.L. Functional and genetic characterization of the LIMD1 gene.

Training Environment

In the past two years the candidate has presented at various internal meetings at the University of Toronto. Among these are the required presentations within the candidates department, Laboratory Medicine and Pathobiology. As well, the Samuel Lunenfeld Research Institute, the location of the candidate's lab, requires students to present their research at weekly seminars attended by faculty and students. He has also presented at the Centre for Cancer Genetics meetings, a monthly meeting of cancer-oriented labs within the Samuel Lunenfeld Research Institute where external feedback is facilitated by the smaller amount of people.

Conclusions

An important accomplishment in the first year of this study was the fine mapping of the region within BRCA2 responsible for the association with TTP. The large size of the original bait (1,300 a.a.) would most likely hinder further biochemical work with these two proteins, but the generation of a shorter BRCA2 region makes future work more manageable. We also showed a one-way *in vivo* association between BRCA2 and TTP under transfected conditions. The second year of this study required a reworking on the original statement of work. This was due to the fact that the interaction failed to repeat subsequent to the submission of the first annual report.

Initially, standard attempts were carried to recreate this association. This included making fresh buffers and ordering new protease inhibitors in case their efficacy had been compromised. These initial procedures failed. Around the time of the interaction cessation, contamination of the incubator forced us to thaw new aliquots of 293 cells. When the new line was established we carried out a coimmunoprecipitation, following the original protocols that generated the initial interaction however, this again failed to produce a positive result. A new aliquot of 293 cells were ordered from ATCC in the event that something had occurred to our frozen aliquots of 293. These new cells did not generate any of the desired interactions. A review of the literature on TTP revealed that TTP is a CRM1 nuclear export dependent protein[10]. TTP, for the most part, is found predominately in the cytoplasm but is however, shuttled in and out of the nucleus. The nuclear export is dependent on the CRM1 transporter, which is inhibited by exposure to LMB. We therefore hypothesized that exposure of 293 cells to LMB may facilitate an interaction between BRCA2 and TTP. This was strengthened by the fact that BRCA2 is found exclusively in the nucleus. Incubation with LMB at various time points failed to recreate this interaction.

We were unable to reproduce the original coimmunoprecipitation results. We attempted to eliminate all possibilities that may have accounted for the cessation of this interaction. It is possible that some "event" occurred within the original culture of 293 cells facilitating the interaction between BRCA2 and TTP, although nothing was observed, at least from a morphological standpoint. This may have accounted for the negative results observed after thawing new batches of 293 cells, as well as ordering new aliquots from ATCC. Unfortunately, this research did not allow us to further examine the proposed interaction. It is an intriguing potential interaction in that it connects BRCA2 (a gene with most of the literature focusing on DNA repair) with the possible control of an extremely potent cytokine, TNF-alpha. In our hands, the ability to repeat the initial result *in vivo* hampered further studies.

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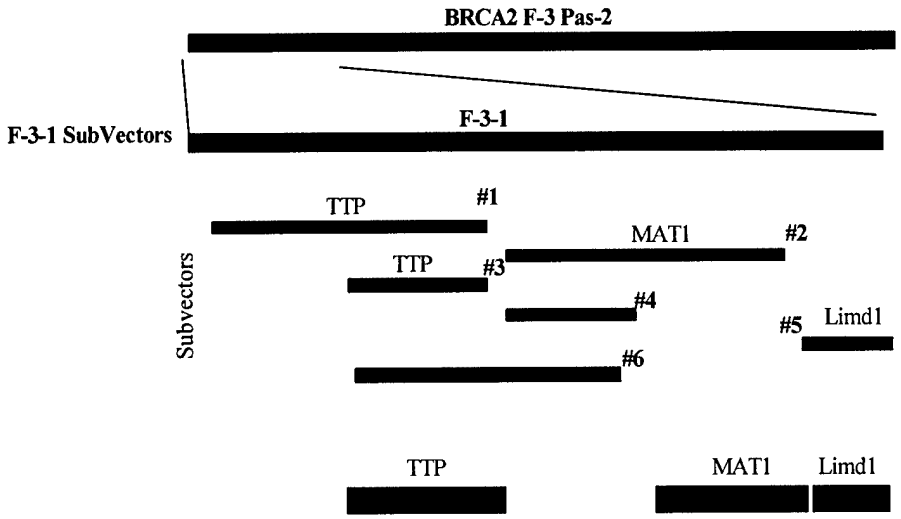
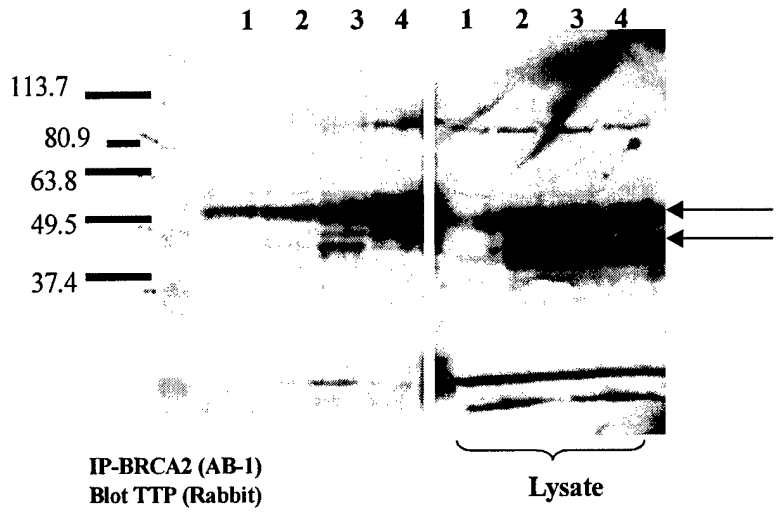


Figure 1a. The original BRCA2 bait (BRCA2 F-3 Pas-2) was subdivided into subvectors (F-3-1) labeled 1 thru 6. TTP was observed to interact with a stretch of BRCA2 corresponding to amino acids 2,177-2,239 (Subvector F-3-1#3). Other potential interacting proteins are also shown for this part of the BRCA2 protein.



Lane1: BRCA2-HA + 2-B (empty vector)
Lane2: BRCA2-HA + 7.5 ug TTP-FL
Lane3: BRCA2-HA + 10 ug TTP-FL
Lane4: BRCA2-HA + 12.5 ug TTP-FL

Figure 1b. Coimmunoprecipitation of TTP with BRCA2 under transfected conditions. Empty vector (Lane 1) and TTP (Lanes 2-4) were transfected at various concentrations in 100mm plates of 293 cells. The multiple phosphorylated levels of TTP are representative of the two major bands (arrows) in the lysate blot. Immunoprecipitating with BRCA2 and blotting with TTP antibodies demonstrated this interaction

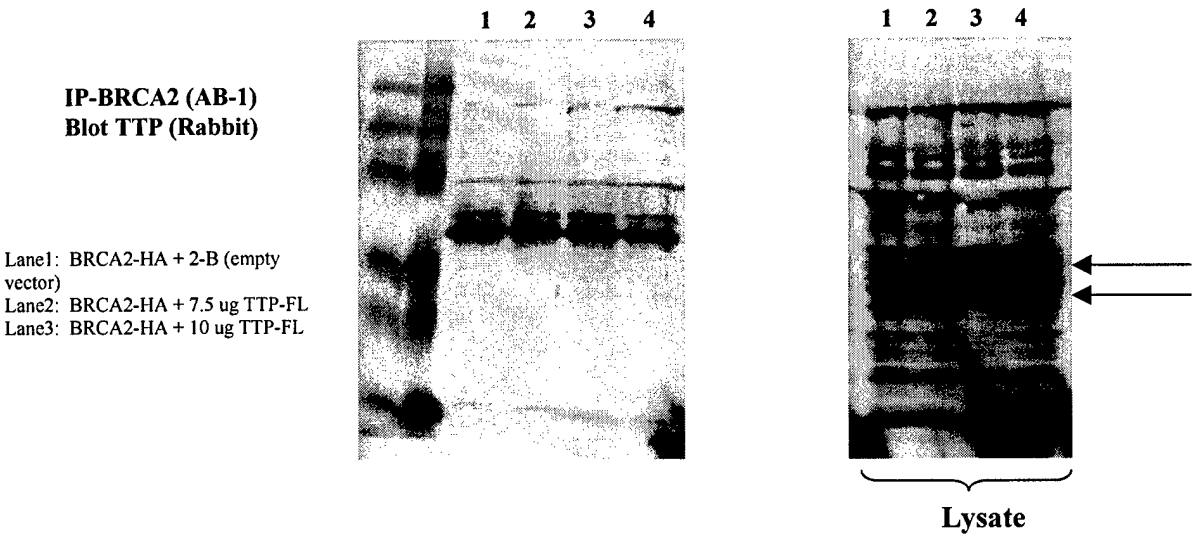


Figure 2a. Coimmunoprecipitation of TTP with BRCA2 under transfected conditions. Empty vector (Lane 1) and TTP (Lanes 2-4) were transfected at various concentrations in 100mm plates of 293 cells. The multiple phosphorylated levels of TTP are representative of the two major bands (arrows) in the lysate blot. Immunoprecipitating with BRCA2 and blotting with TTP antibodies failed to demonstrate the intial co-immunoprecipitation.

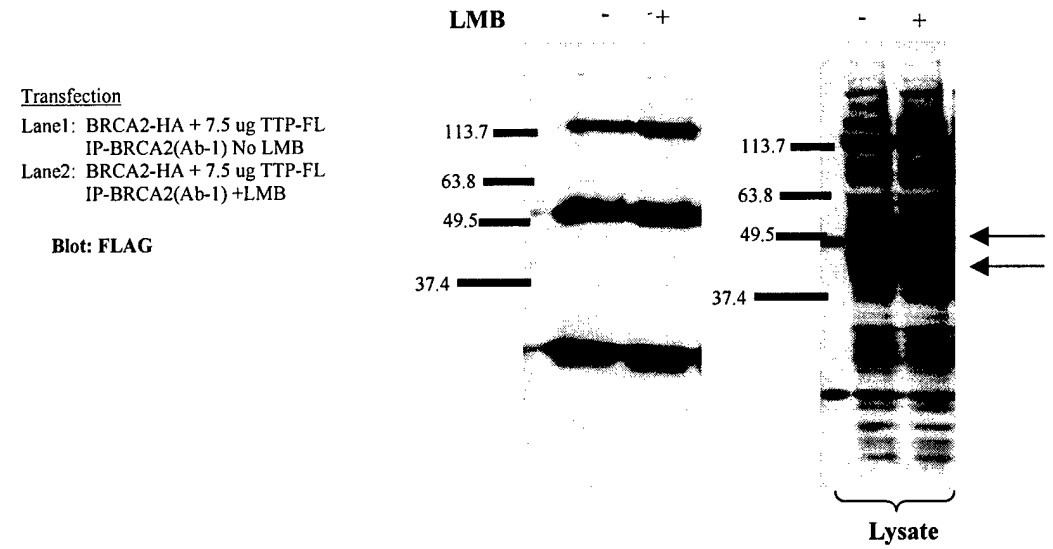


Figure 2b. Negative coimmunoprecipitation results with and without 10ng/mL LMB incubation. Lanes 1 and 2 on the lysate blot show successful transfection of TTP in 100mm plates of 293 cells indicated by the thick bands (arrows) between the 36-50kDa range. The lack of corresponding TTP bands in the coimmunoprecipitation blot (left blot, under the heavy chain bands at 55kDa). Cells were mock incubated (DMSO) or incubated with 10ng/mL of LMB.

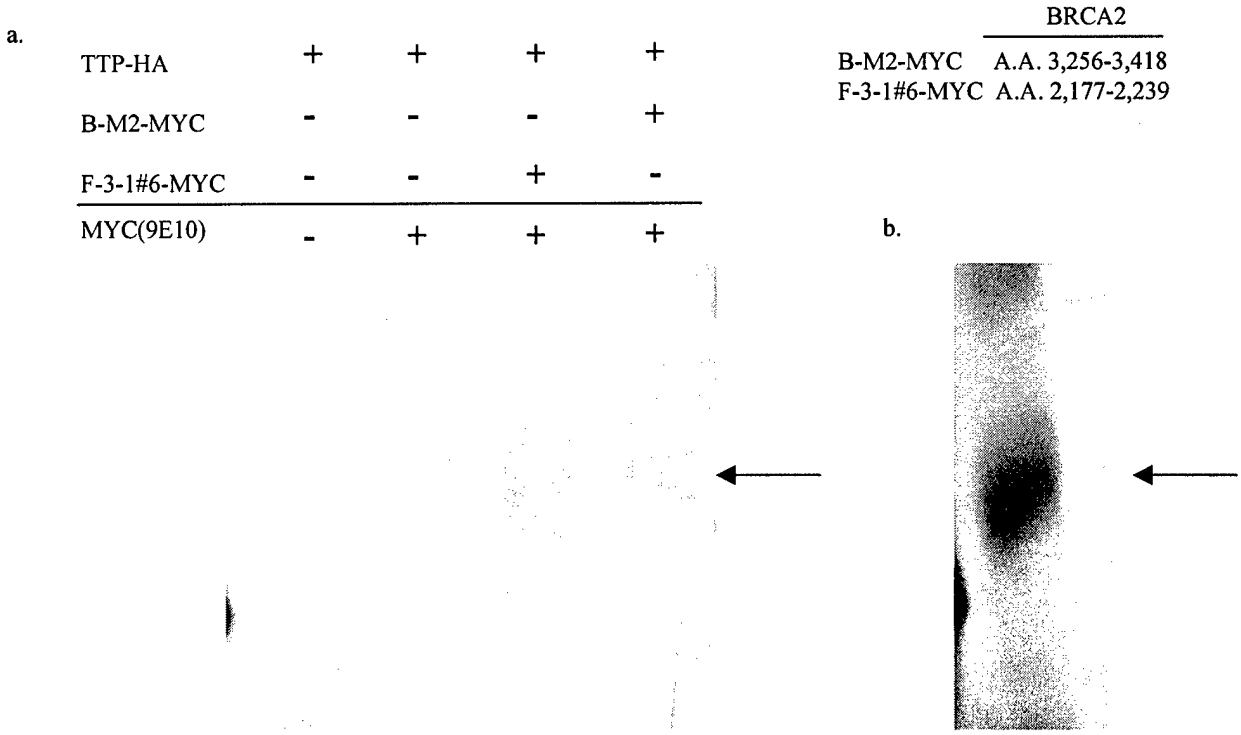


Figure 3a. *In vitro* S^{35} analysis of BRCA2 and TTP. *In vitro* translated BRCA2 peptides B-M2-MYC and F-3-1#6-MYC were incubated with either Protein G (Lane1), MYC(9E10) antibody(Lanes 2-4), and 10uL of TTP-HA(Lanes 1-4). 10uL of TTP-HA lysate is shown in "b" (arrow). Coimmunoprecipitation is observed in lanes 3 and 4 in "a".